Supplementary Materials for

Host Genetic Diversity Enables Ebola Hemorrhagic Fever Pathogenesis and Resistance

Angela L. Rasmussen, Atsushi Okumura, Martin T. Ferris, Richard Green, Friederike Feldmann, Sara M. Kelly, Dana P. Scott, David Safronetz, Elaine Haddock, Rachel LaCasse, Matthew J. Thomas, Pavel Sova, Victoria S. Carter, Jeffrey M. Weiss, Darla R. Miller, Ginger D. Shaw, Marcus J. Korth, Mark T. Heise, Ralph S. Baric, Fernando Pardo-Manuel de Villena, Heinz Feldmann, and Michael G. Katze.

correspondence to: honey@uw.edu

This PDF file includes:

Materials and Methods
SupplementaryText
Figs. S1 to S9
Captions for TableS1 to S3

Other Supplementary Materials for this manuscript includes the following:

Databases S1 to S3 as zipped archives:
Table S1
Table S2
Table S3

Materials and Methods

Biosafety statement. All infectious work with WT-EBOV and MA-EBOV was approved by the Rocky Mountain Laboratories (RML) Institutional Biosafety Committee (IBC), and performed in a biosafety level-4/animal biosafety level-4 (BSL-4/ABSL-4) high containment facility at RML Division of Intramural Research (DIR), National Institute of Allergy and Infectious Diseases (NIAID), National Institutes of Health (NIH). IBC-approved standard operating procedures were applied for all infectious work.

Animals. C57BL/6J, A/J, 129S1/SvImJ, NOD/ShiLtJ, NZO/H1LtJ, CAST/EiJ, PWK/PhJ, and WSB/EiJ mice were obtained from The Jackson Laboratory. The Systems Genetics Core Facility (University of North Carolina) provided susceptible 13140x3015 and resistant 15156x1566 mice, which were produced by crossing OR13140 dams with OR3015 sires, and OR15156 dams with OR1566 sires. All CC-RIX lines used in this study were generated in this way, and full information about each CC genome can be found at the CC website (http://csbio.unc.edu/CCstatus). All mice were sent directly to the Integrated Research Facility (IRF) at RML, and acclimated for five days in ABSL-4 conditions prior to infection. All experiments were conducted on six-to-eight week old male mice. All animal experiments were approved by the RML Institutional Animal Care and Use Committee (IACUC) and carried out in compliance with the NIH Guide for the Care and Use of Laboratory Animals and guidelines established by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC) by certified staff in an AAALAC-approved facility. Groups of a minimum of three CC-RIX mice per timepoint per experiment were inoculated by intraperitoneal injection with 10^2 focus forming units (FFU) MA-EBOV or an equivalent volume of DMEM with 2% FBS. This sample size was selected based on previous experience with mouse pathogenesis studies and CC-RIX animal availability, and animals were assigned randomly to infection or mock groups in a non-blinded manner.

Virus and cells. Vero E6 cells (African green monkey kidney cells) were maintained at 37° C with 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum (FBS), 50 U/mL penicillin, and 50 µg/mL streptomycin. WT-EBOV (Mayinga strain) and MA-EBOV were amplified in Vero E6 cells using DMEM supplemented with 2% FBS as previously described(*1*, *2*). Virus infectivity titers (FFU) were determined by indirect immunofluorescent staining of serially diluted virus stock or organ homogenates from infected animals in triplicate on confluent Vero E6 cells in a 96-well plate. Following adsorption of inoculum, cells were overlaid with Eagle's minimal essential medium (MEM) with 3% FBS and 1.5% carboxymethylcellulose. Cells were incubated for 5 days at 37°C, 5%CO_2. The overlay was removed by washing with phosphate buffered saline (PBS), and cells were fixed in 10% formalin. Plates were treated for 30 minutes with 0.25% Triton X-100, washed thrice with PBS, and immunostained. Cells were stained with mouse anti-EBOV VP40(*3*) at a 1:10,000 concentration for 2 hours, washed thrice with PBS, and incubated with FITC-conjugated secondary antibody (Sigma-Aldrich). Foci were then enumerated using a fluorescence microscope.

Sample collection from infected animals. Liver and spleens were collected at necropsy following humane euthanasia by isoflurane overdose or exsanguination while under deep anesthesia. Samples were divided into three equally sized portions. One portion was formalin fixed for histopathology. One portion was weighed and placed in 0.5 mL PBS and frozen prior to homogenization and centrifugation for virus infectivity titration. One portion was weighed and submerged in 10-20 volumes RNAlater (Life Technologies) RNA stabilization reagent, stored overnight at 4° C, then frozen at -80° C until homogenization in RNA extraction buffer for RNA isolation. Blood samples for coagulation analysis were collected by cardiac puncture with a 26-gauge needle and 1 mL syringe pretreated with 0.1 mL of 10.5 mM sodium citrate tribase. Blood samples were immediately analyzed as described below.

Coagulation analysis. Blood clotting times and serum fibrinogen concentration were determined using the Stago Diagnostica STart4 Hemostasis Analyzer with accompanying reagents per the manufacturer's instructions. Samples were excluded from analysis if insufficient volumes of blood were present in the sample or if the animal succumbed to lethal shock prior to exsanguination.

Histopathology. Tissues were fixed in 2 changes of 10% neutral buffered formalin, for a minimum of 7 days. Tissues were placed in cassettes and processed with a Sakura VIP-5 Tissue Tek, on a 12 hour automated schedule, using a graded series of ethanol, xylene, and ParaPlast Extra. Embedded tissues were sectioned at 5 μm and dried overnight at 42° C prior to staining. All tissue sections were stained with hematoxylin and eosin (H&E). Specific anti-EBOV immunoreactivity was detected using EBOV VP40 polyclonal rabbit antisera at a 1:2000 dilution. Specific anti-caspase-3 immunoreactivity was detected using anti-active caspase 3 polyclonal rabbit antibody (Promega) at a 1:50 dilution. Tissues were then processed for immunohistochemistry using the Discovery XT automated processor (Ventana Medical Systems) with a DAPMap (Ventana Medical Systems) kit. Terminal dUTP nick end labeling (TUNEL) analysis was preformed with the ApopTag Plus Peroxidase *In Situ* Apoptosis Detection Kit from (EMD Millipore) using the peroxidase staining for paraffin-embedded tissue protocol.

RNA isolation. Liver and spleen tissues collected for RNAisolation were directly submerged in RNAlater stabilization solution following dissection, placed at 4°C overnight, and frozen at −80°C as described above. Tissues were thawed, transferred to TRIzol (Life Technologies) or RLT Reagent (Qiagen Inc.) and homogenized. RNA was isolated using Qiagen miRNeasy columns and the manufacturer's recommended protocol. RNA quality was assessed on an Agilent 2100 Bioanalyzer using the nanochip, which was used to exclude samples not meeting quality or yield criteria.

RT-PCR quantification of MA-EBOV RNA. Viral genomes were quantified from liver and spleen RNA using reverse transcription polymerase chain reaction (RT-PCR). The QuantiTect reverse transcription kit (Qiagen) was used to generate cDNA. Quantitative real-time RT-PCR (qRT-PCR) was run on an ABI 7900 PCR system. A

custom Taqman assay designed to amplify sequence of NP from both WT-ZEBOV and MA-ZEBOV was obtained from Life Technologies and used the following primer-probe sequences: NP forward 5'-TCATGGCAATCCTGCAACA-3', NP reverse 5'-TCGGTTGAATCATCCCATTGT-3', NP probe 6FAM-5'-CATCAGTGAATGAGCATGG-3'-MGBFNQ. 18S ribosomal RNA content assayed with forward 5'- GAACGTCTGCCCTATCAACTTTC -3' and reverse 5'-GATGTGGTAGCCGTTTCTCAG - 3' primers was used as an endogenous control to calculate relative expression. Viral genomes are represented as $\log_{10}$ relative quantification, using the 2-ΔΔCT method.

Affymetrix Target Preparation and Microarray Hybridization. RNA samples were prepared for whole transcriptome expression analysis using the WT PLUS Reagent Kit following the manufacturer's recommended protocol (Affymetrix, Inc.). 100 ng of total RNA was used to prepare the hybridization ready targets. Individual sense-strand DNA targets were randomized and hybridized to Mouse Gene 2.1 ST 24-Array Plates (Affymetrix, Inc.) using the GeneTitan Multi-Channel (MC) Instrument for hybridization, array staining and washing and scanning. Quality control (QC) metrics for hybridization, labeling, and sample quality were generated using the Affymetrix Expression Console (version 1.3.187) software. All samples passed the QC criteria.

Transcriptomic analysis. To identify outliers to exclude from the analysis, samples were screened using the Affymetrix expression console with box plots, as well as multi dimensional scaling (MDS) and inter-array correlation (IAC) plots using the R statistical programming language (version 3.01). The data was analyzed in R using Bioconductor (version 2.13) and packages oligo (1.26.6) and limma (3.18) and their dependencies. Background correction, normalization, and probe summarization were performed with RMA (Robust Multichip Average) in the limma package. Probes were masked using a custom probe masking script (see description below). The Combat package was run to correct for plate batch effects(*4*). Differential expression analysis was performed using the limma package. Lists of differentially expressed genes (DEG) were generated for each time point post-infection based on fold change > |1.5| and FDR-corrected p-value <0.05. Ingenuity Pathway Analysis (IPA) software (Ingenuity Systems) was used for functional analysis of DE genes. IPA Molecular Activity Prediction function was used to identify build a network showing TIE1 and TEK interactions with other related molecules and predict activity. Blood Coagulation genes were identified using IPA's canonical pathways, and one-dimensional hierarchical clustering was performed with Spotfire DecisionSite 9.1 (Tibco), using the unweighted average method (Unweighted Pair Group with Arithmetic Mean). Microarray data has been deposited with the Gene Expression Omnibus (www.ncbi.nlm.nih.gov/geo) (accession number GSE57214).

CC-specific custom probe masking. All raw Affymetrix probe sequences were downloaded from the NetAffx website (www.affymetrix.com/analysis/index.affx). All probes were mapped using Bowtie2. Bowtie2 mapping parameters were set to an exact length of 25 (length of probe sequence) and allowed for one mismatch.

Bowtie2 mapping was performed against all eight founders. Founder Bowtie2 indexes were created using pseudogenomes available through the Systems Genetics Core Facility website (www.csbio.unc.edu/CCStatus/index.py)(*5*). All probe sequences were then mapped against all eight CC founder genomes. All the mapped probe sequences were parsed using Linux bash scripts and loaded into the R statistical programming environment. Within R, all sequences that matched consistently across the intersection of all eight founders were considered and used as masking criteria. This resulted in the selection of 35648 out of 41345 total probe sets for inclusion in the analysis.

Sequencing of viral genomes. Whole transcriptome libraries were constructed using the TruSeq Stranded Total RNA with Ribo-Zero Gold (Illumina) according to the manufacturer's guide. Libraries were quality controlled and quantitated using the BioAnalzyer 2100 system and qPCR (Kapa Biosystems). Libraries were enriched for sequences of mouse-adapted Zaire ebolavirus(*6*) using Agilent SureSelect probes designed for the full length genome. Libraries containing enriched Ebola sequence were sequenced on a MiSeq (Illumina) at a cluster density of approximately (1200K)/mm^2 using v3 reagents. Sequence was quality controlled using Illumina's MiSeq Controller Software version 2.4.0.4. Any remaining ribosomal or mouse reads were removed by mapping using Bowtie(*7*). Remaining reads were assembled and variants assessed using CLC GenomicsWorkBench 7 (Qiagen)

Statistical analyses. Statistical analyses for virus replication data were calculated using unpaired t-tests in Graphpad Prism 6. Statistical analyses for coagulation analyses were calculated using Box-Cox transformed data, and ANOVA and Tukey's HSD post-hoc test in the R statistical programming language. FDR-adjusted p-values < 0.05 were considered significant. Statistical analyses for sequencing and microarray experiments were performed as described above.

Fig. S1. Survival of CC Founders Infected with MA-EBOV and WT-EBOV.
Kaplan-Meier survival curve for C57BL/6J (A), A/J (B), 129S1/SvlmJ (C), NOD/ShiLtJ (D), NZO/H1LtJ (e), CAST/EiJ (f), PWK/PhJ (g), and WSB/EiJ (h). Red lines indicate inoculation with 10 FFU MA-EBOV, blue lines indicate inoculation with 10^4 FFU MA-EBOV, and green lines indicate inoculation with 10^4 FFU WT-EBOV. Four mice were used per dose group per founder line.

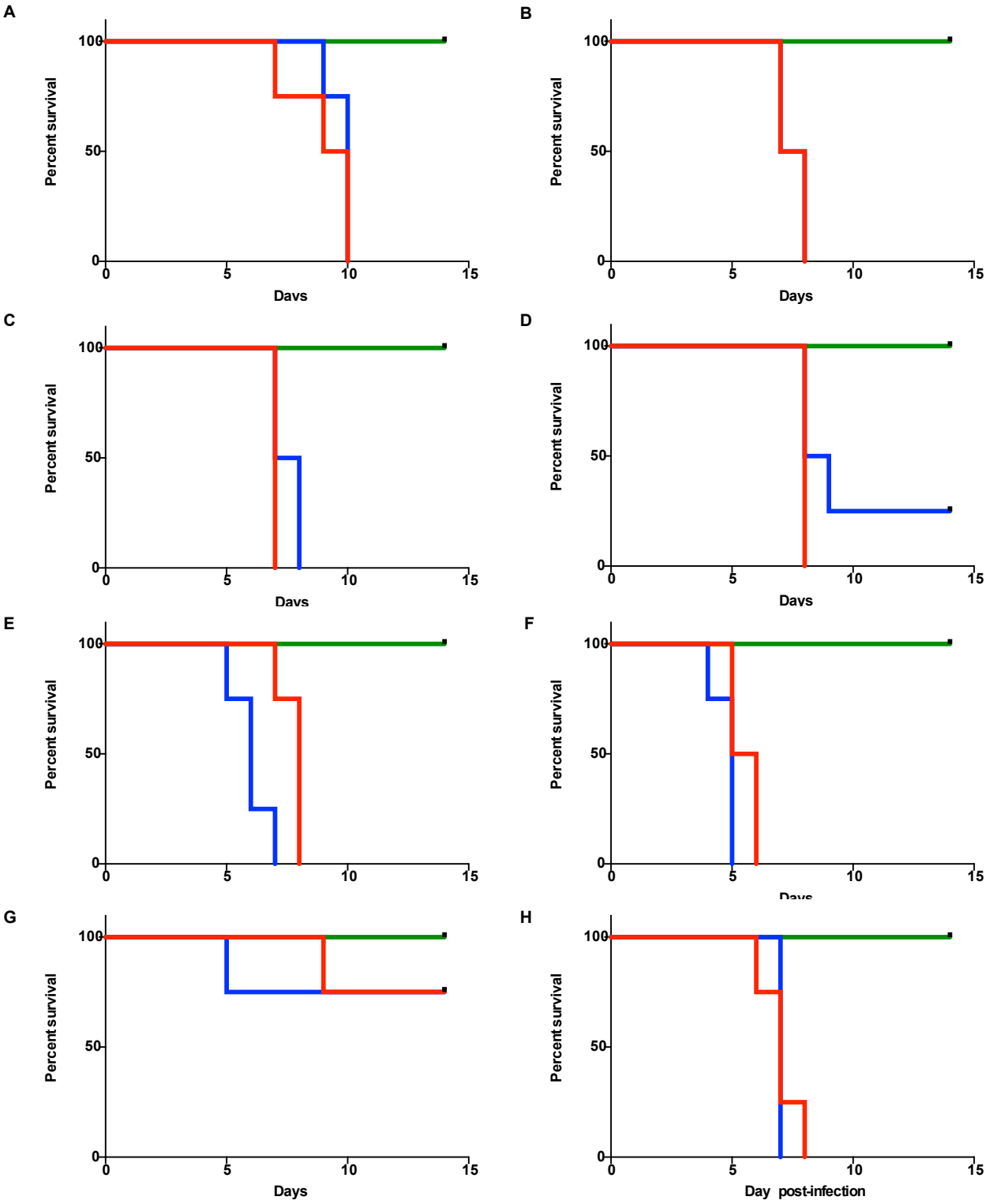
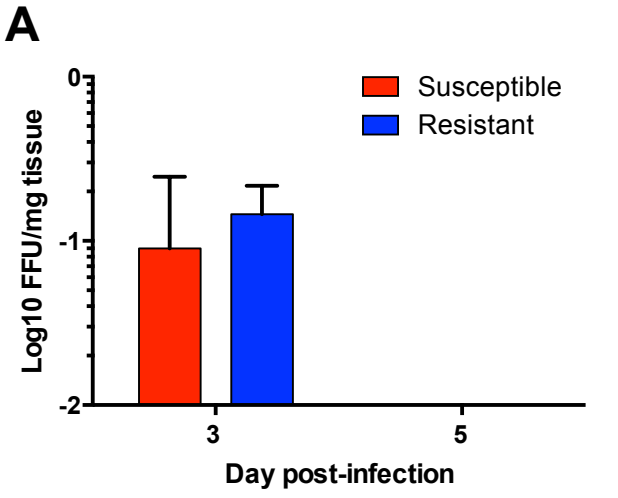

A
B
C
D
E
F
G
H
Percent survival
100
50
0
0
5
10
15
Days
Day post-infection

Fig. S2. Organ Titers of WT-EBOV in CC-RIX Mice. Virus titer determined by focus forming assay in the spleen (A) or liver (B) of susceptible (red) or resistant (blue) CC-RIX mice.


A
Susceptible
Resistant
0
-1
-2
Log10 FFU/mg tissue
3
5
Day post-infection

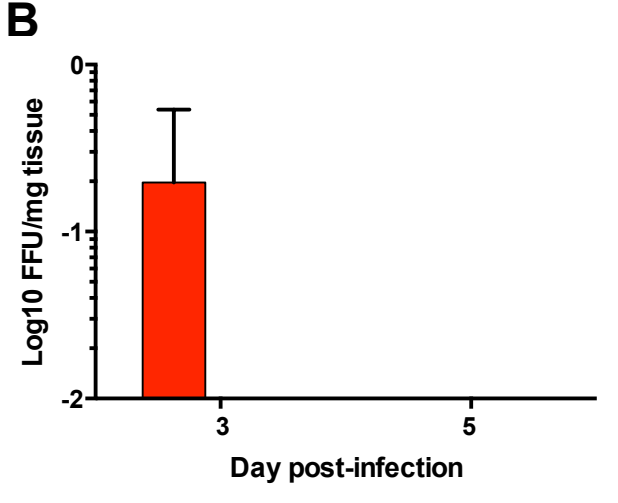

B
0
-1
-2
Log10 FFU/mg tissue
3
5
Day post-infection

Fig. S3. Viral Antigen Distribution in Target Tissues. Immunostaining of MA-EBOV VP40 in susceptible mock-infected spleen (A,B) and liver (M,N), on day 3 p.i. in spleen (E,F) and liver (Q,R), and on day 6 p.i. in spleen (I,J) and liver (U,V), and resistant mock-infected spleen (C,D) and liver (O,P), on day 3 p.i. in spleen (G,H) and liver (S,T), and on day 6 p.i. in spleen (K,L) and liver (W,X).

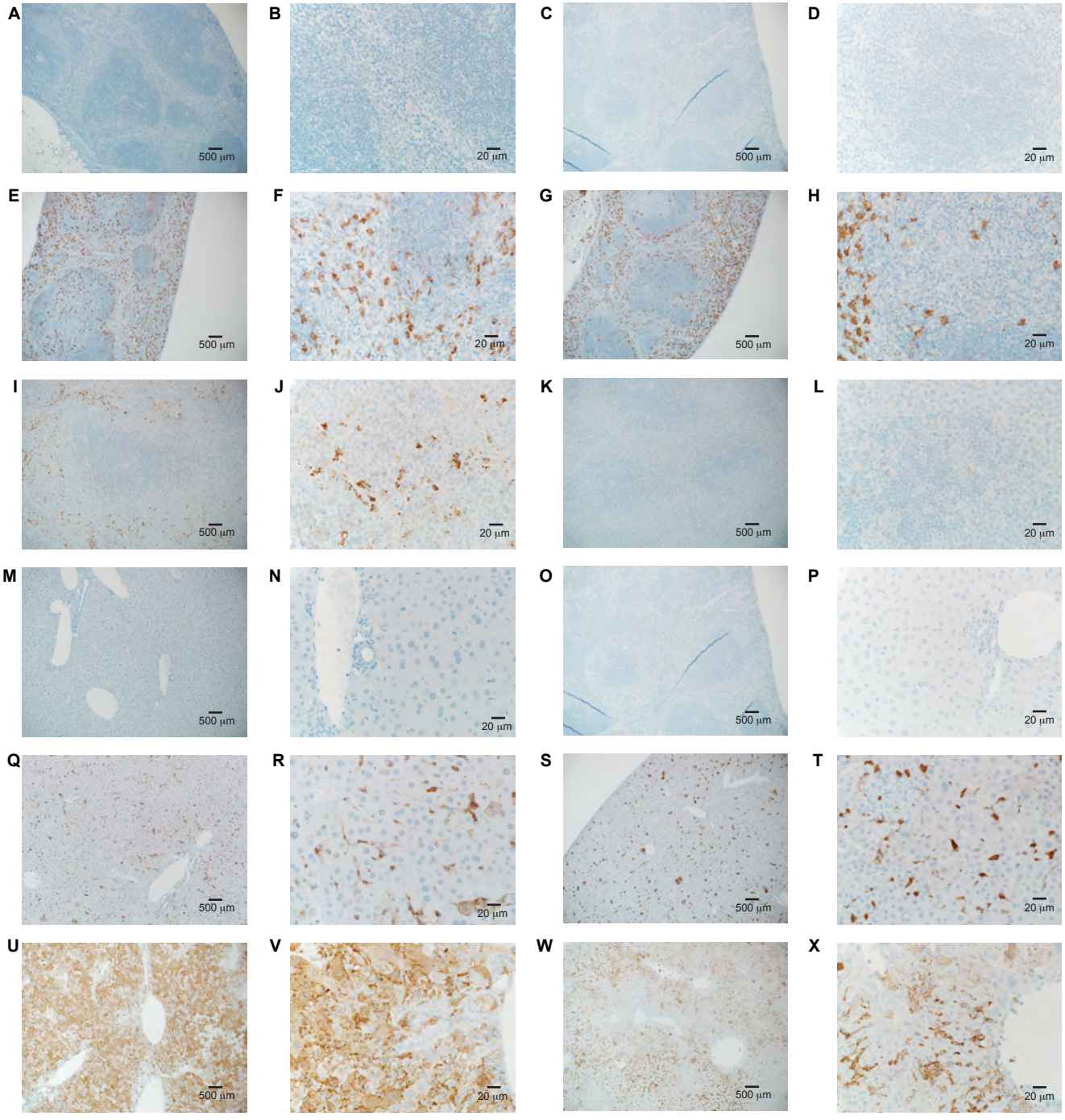

A
500 μm
B
20 μm
C
500 μm
D
20 μm
E
500 μm
F
20 μm
G
500 μm
H
20 μm
I
500 μm
J
20 μm
K
500 μm
L
20 μm
M
500 μm
N
20 μm
O
500 μm
P
20 μm
Q
500 μm
R
20 μm
S
500 μm
T
20 μm
U
500 μm
V
20 μm
W
500 μm
X
20 μm

Fig. S4. Histopathologic Assessment of Inflammation in Target Tissues. H&E staining in susceptible mock-infected spleen (A,B) and liver (M,N), on day 3 p.i. in spleen (E,F) and liver (Q,R), and on day 6 p.i. in spleen (I,J) and liver (U,V), and resistant mock-infected spleen (C,D) and liver (O,P), on day 3 p.i. in spleen (G,H) and liver (S,T), and on day 6 p.i. in spleen (K,L) and liver (W,X).

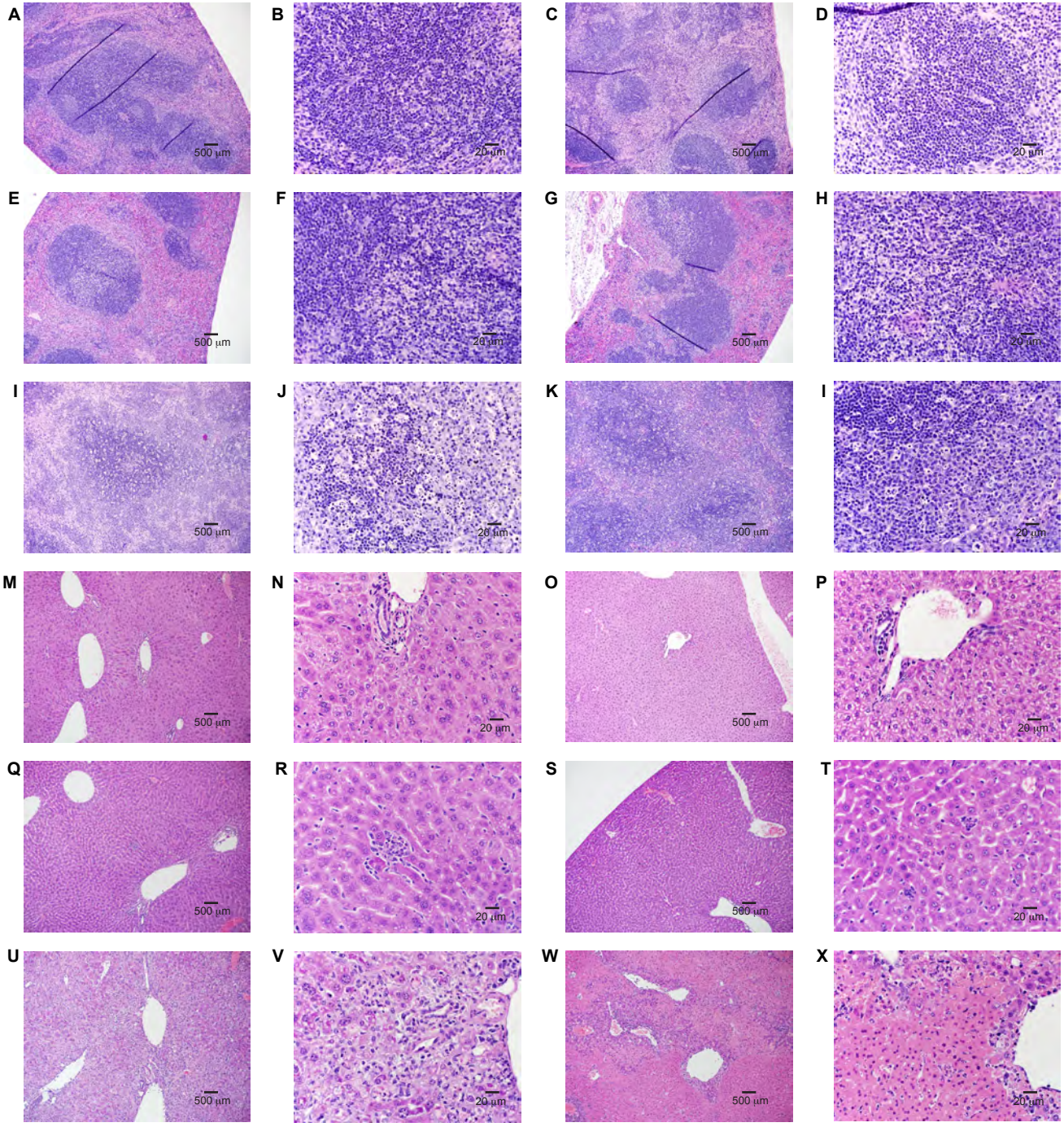

A
500 μm
B
20 μm
C
500 μm
D
20 μm
E
500 μm
F
20 μm
G
500 μm
H
20 μm
I
500 μm
J
20 μm
K
500 μm
I
20 μm
M
500 μm
N
20 μm
O
500 μm
P
20 μm
Q
500 μm
R
20 μm
S
500 μm
T
20 μm
U
500 μm
V
20 μm
W
500 μm
X
20 μm

Fig. S5. Apoptosis in Target Tissues. TUNEL staining in susceptible mock-infected spleen (A,B) and liver (I,J) and on day 6 p.i. in spleen (E,F) and liver (M,N), and resistant mock-infected spleen (C,D) and liver (K,L) and on day 6 p.i. in spleen (G,H) and liver (O,P).

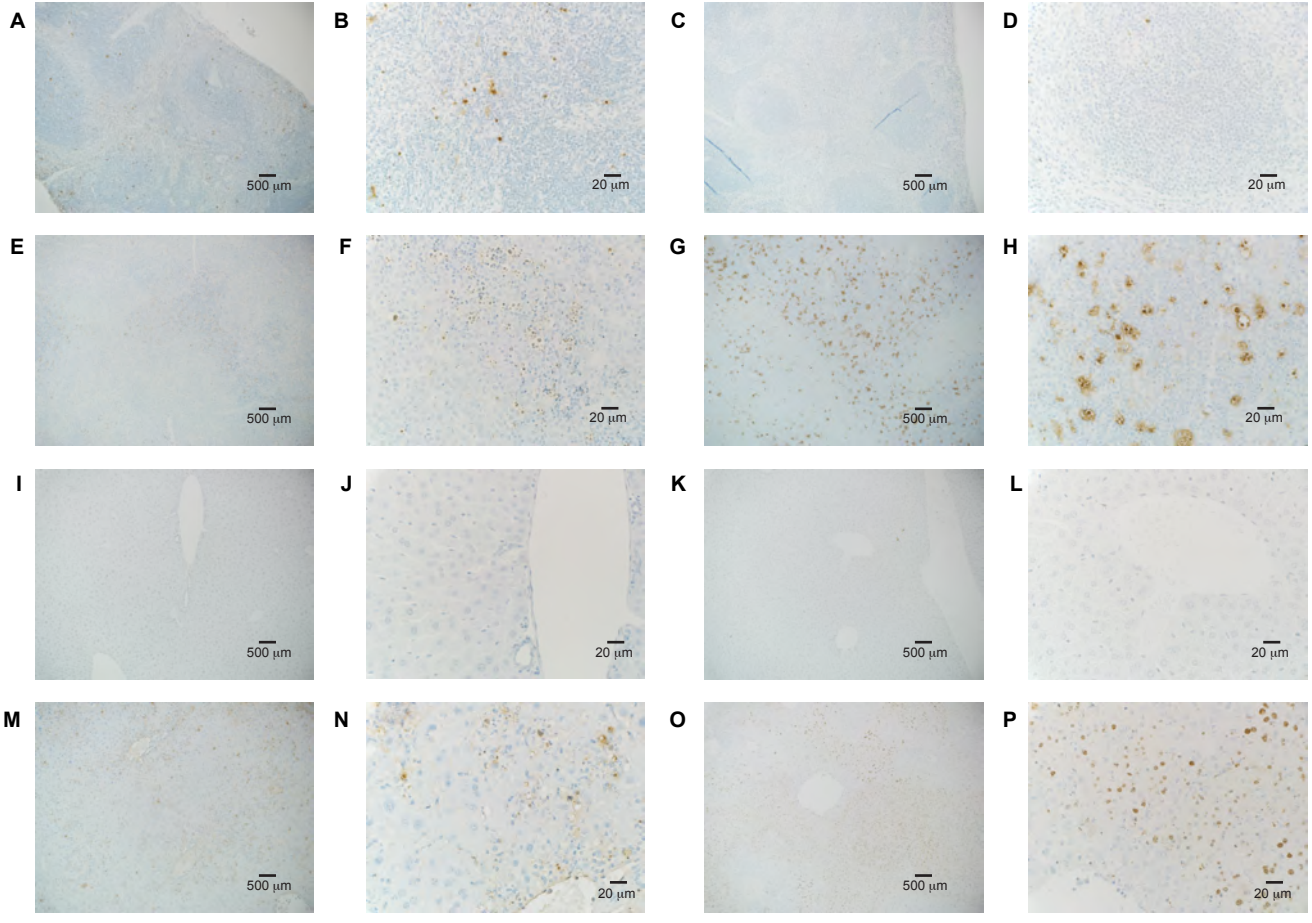

A
500 μm
B
20 μm
C
500 μm
D
20 μm
E
500 μm
F
20 μm
G
500 μm
H
20 μm
I
500 μm
J
20 μm
K
500 μm
L
20 μm
M
500 μm
N
20 μm
O
500 μm
P
20 μm

Fig. S6. Caspase-3 Activation in Target Tissues. Immunostaining for activated caspase-3 in susceptible mock-infected spleen (A,B) and liver (I,J) and on day 6 p.i. in spleen (E,F) and liver (M,N), and resistant mock-infected spleen (C,D) and liver (K,L) and on day 6 p.i. in spleen (G,H) and liver (O,P).

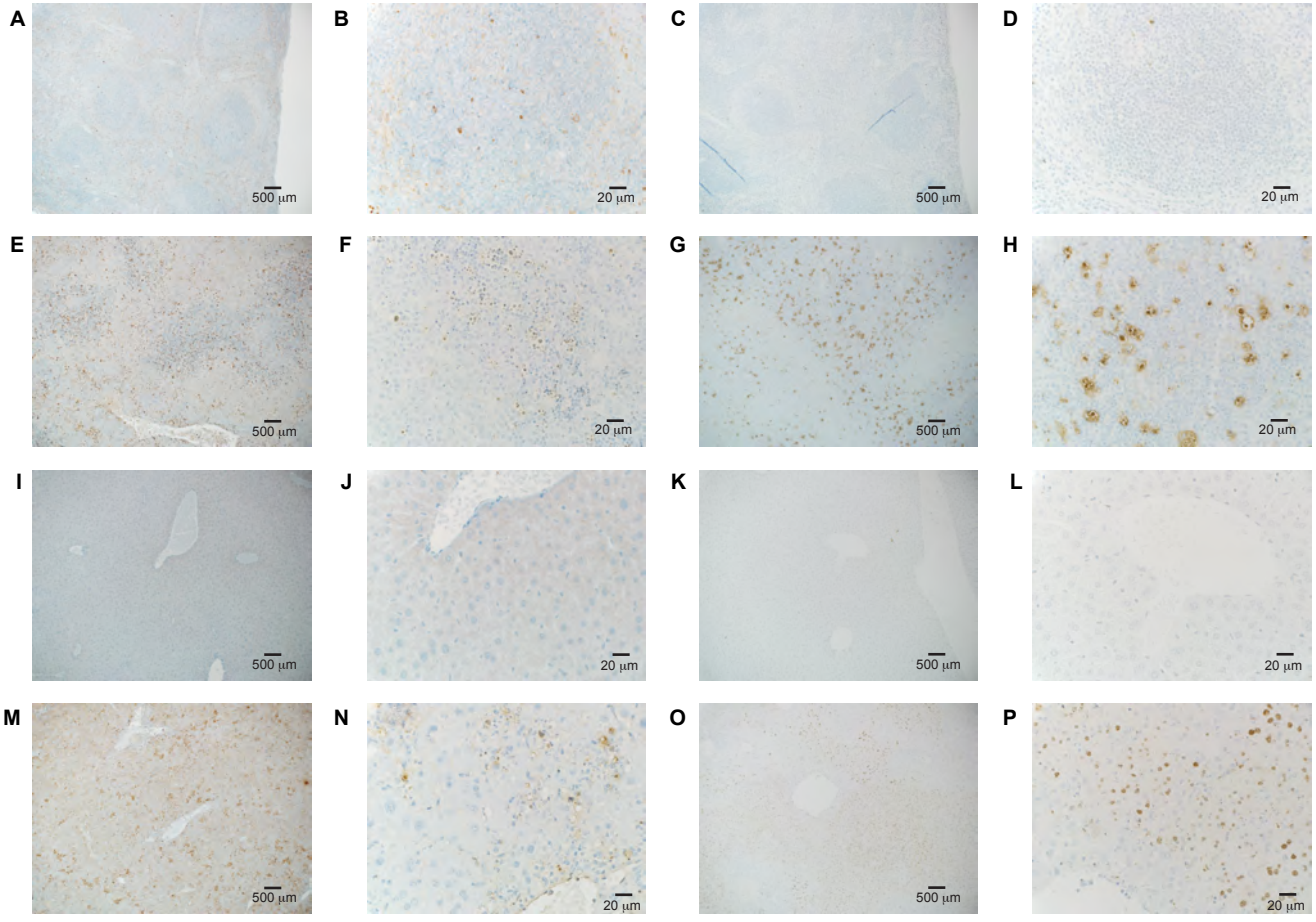

A
500 μm
B
20 μm
C
500 μm
D
20 μm
E
500 μm
F
20 μm
G
500 μm
H
20 μm
I
500 μm
J
20 μm
K
500 μm
L
20 μm
M
500 μm
N
20 μm
O
500 μm
P
20 μm

Fig. S7. Predicted Activity of TIE1 and TEK Signaling in Susceptible CC-RIX Mouse Livers. Network generated by IPA Molecule Activity Prediction software showing molecules associated with TIE1, TEK, and IL-6 in susceptible infected mouse livers at day 3 and day 5 p.i., with either $\log_2$ ratio data (up-regulation: red molecules, down-regulation: cyan molecules) or predicted activity (activation: orange, inhibition: blue) overlays. Connecting lines show predicted activating (orange) or inhibitory (blue) effects on target molecules in the network. Molecules are grouped spatially according to their known biological functions.

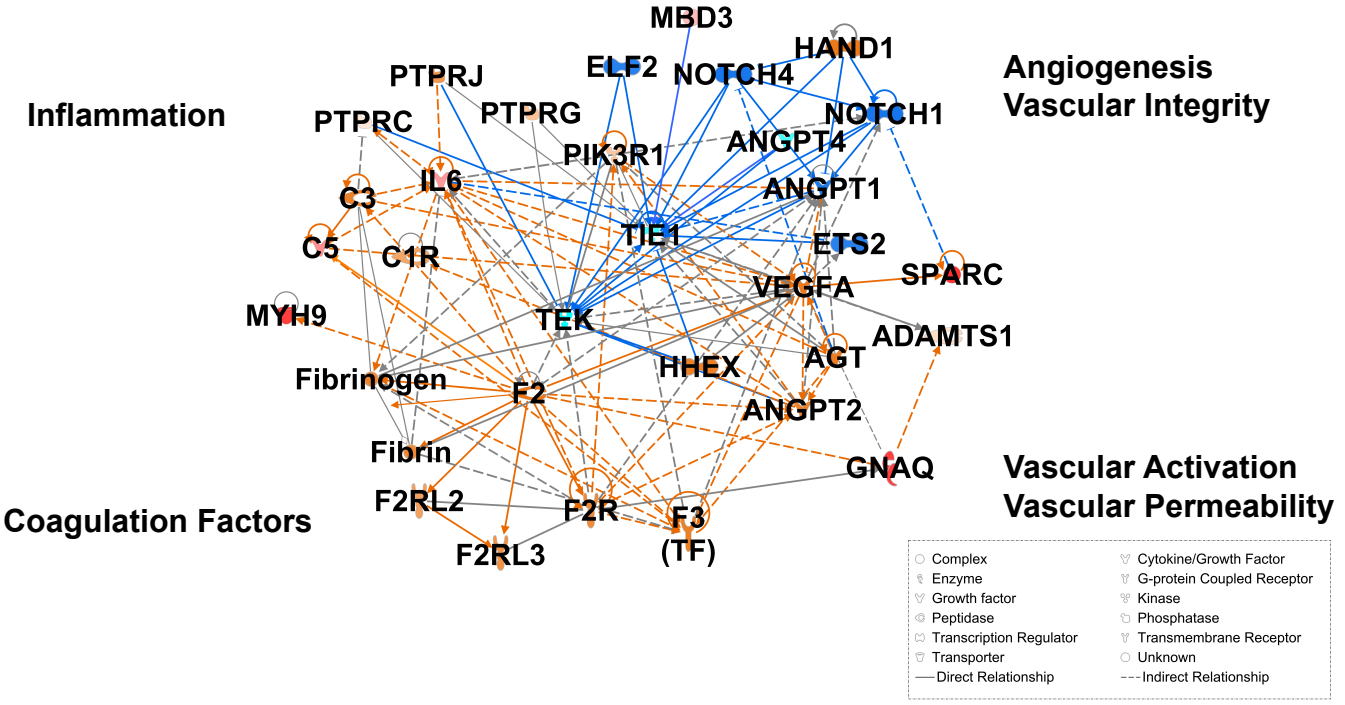

Inflammation
Coagulation Factors
Angiogenesis
Vascular Integrity
Vascular Activation
Vascular Permeability
MBD3
HAND1
ELF2
NOTCH4
PTPRJ
PTPRC
PTPRG
NOTCH1
PIK3R1
ANGPT4
IL6
C3
ANGPT1
C5
C1R
TIE1
ETS2
SPARC
VEGFA
MYH9
TEK
ADAMTS1
AGT
HHEX
Fibrinogen
F2
ANGPT2
Fibrin
GNAQ
F2RL2
F2R
F3
(TF)
F2RL3
Complex
Enzyme
Growth factor
Peptidase
Transcription Regulator
Transporter
Direct Relationship
Cytokine/Growth Factor
G-protein Coupled Receptor
Kinase
Phosphatase
Transmembrane Receptor
Unknown
Indirect Relationship

Fig. S8. Heatmap Showing Genes Involved in Blood Coagulation. Hierarchical clustering of transcripts categorized in the IPA "Blood Coagulation" canonical pathway in susceptible or resistant mouse liver relative to time-matched mock-infected animals.

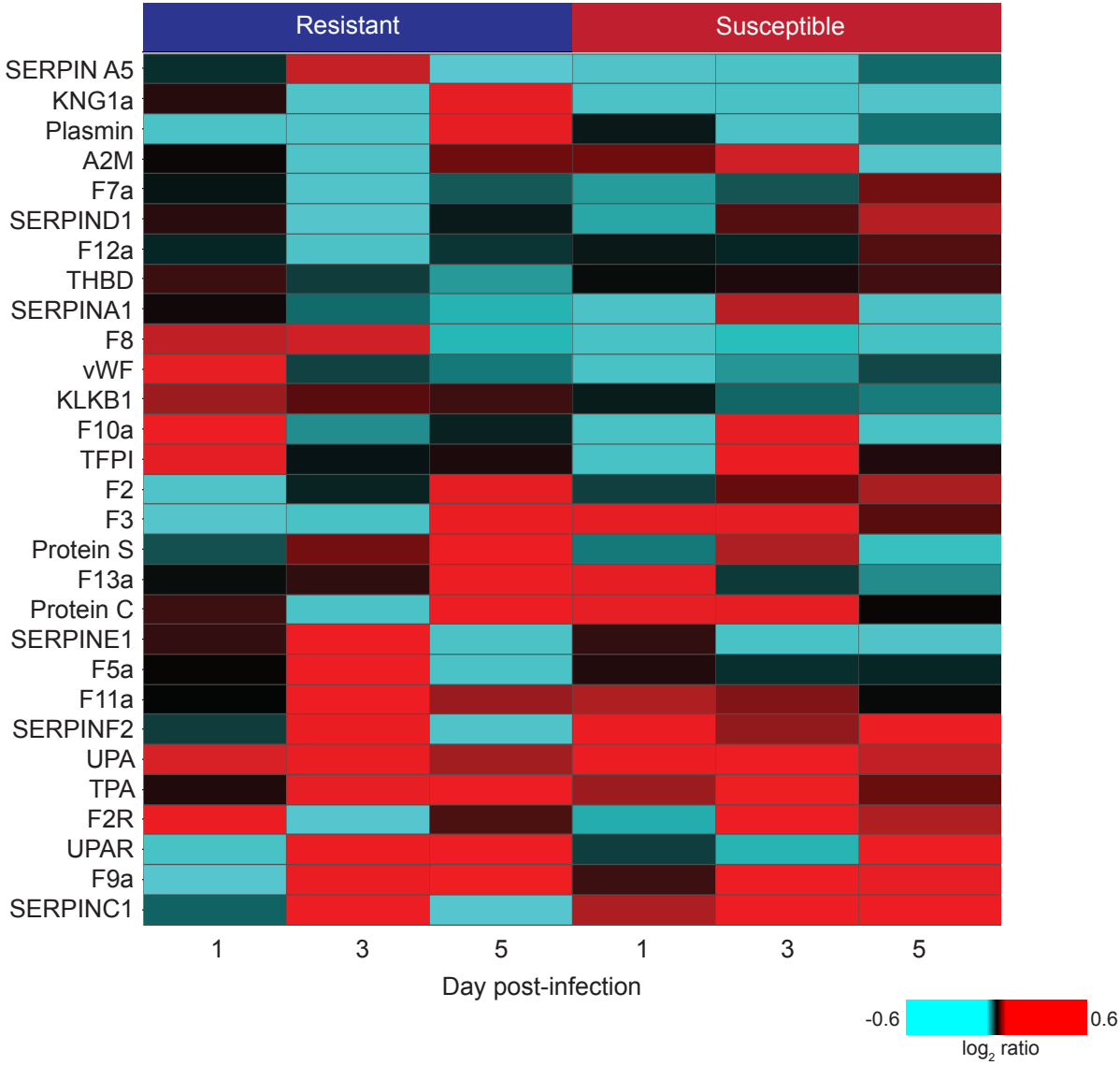
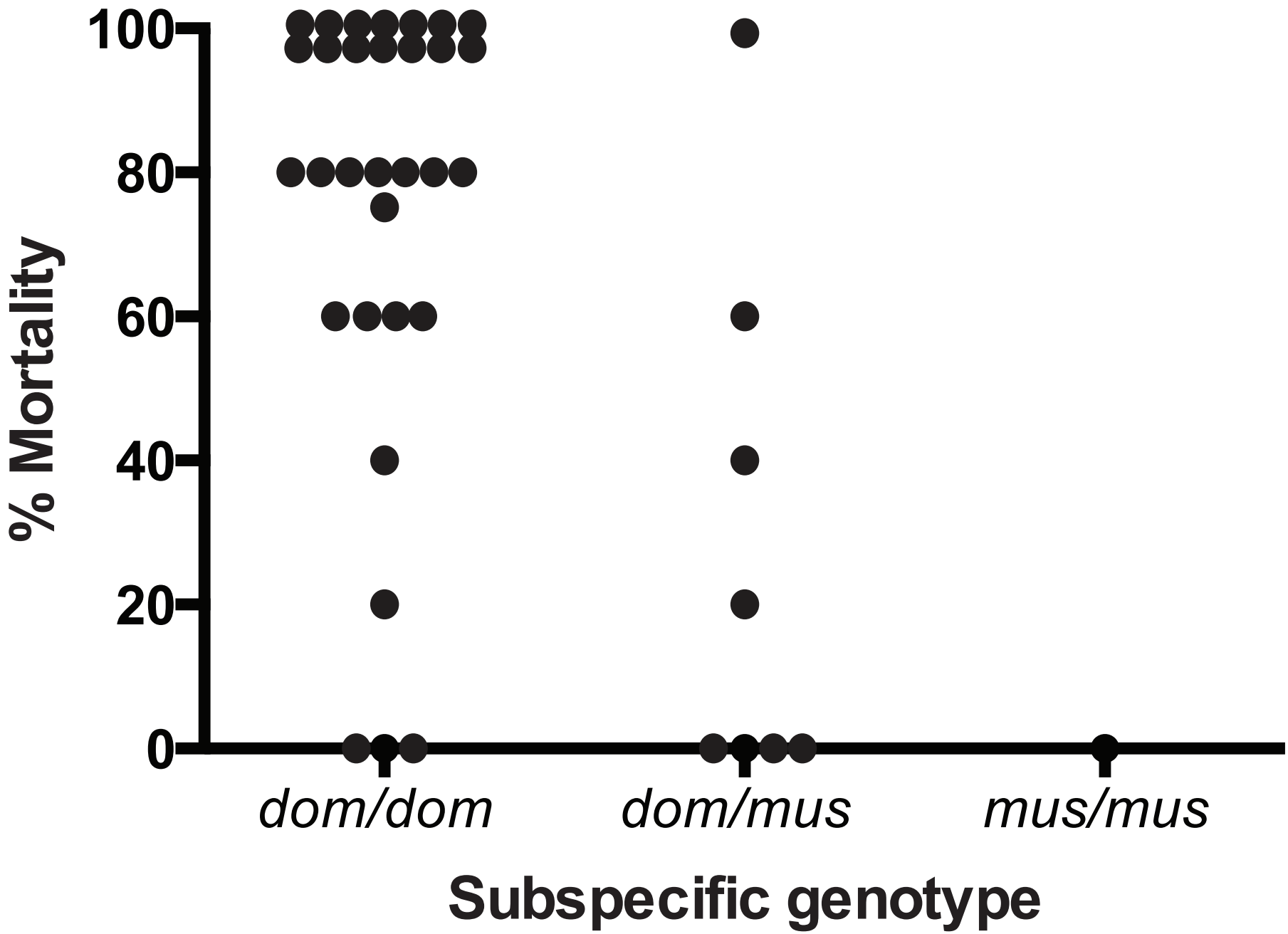

Resistant
Susceptible
SERPIN A5
KNG1a
Plasmin
A2M
F7a
SERPIND1
F12a
THBD
SERPINA1
F8
vWF
KLKB1
F10a
TFPI
F2
F3
Protein S
F13a
Protein C
SERPINE1
F5a
F11a
SERPINF2
UPA
TPA
F2R
UPAR
F9a
SERPINC1
1
3
5
1
3
5
Day post-infection
-0.6
0.6
log2 ratio

Fig. S9. Subspecific Origin of *Tek* Allele in CC-RIX Associated with Mortality. Graph showing association of percent mortality induced by MA-EBOV infection with subspecific origin of the *Tek* allele (homozygous *M. m. musculus*, homozygous *M. m. domesticus*, heterozygous). Each black circle signifies a CC-RIX line screened.


100
80
60
40
20
0
% Mortality
dom/dom
dom/mus
mus/mus
Subspecific genotype

Additiional Data table S1 (separate file)

Table S1. Viral variants identified by RNAsequencing. Workbook tabs show sequence of viral RNA reads mapped to EBOV sequences specified by NCBI accession number.

Additiional Data table S2 (separate file)

Table S2. Significantly differentially expressed genes in spleen. Workbook tabs indicate conditions for lists of DEG from spleen (FDR-corrected p-value < 0.05, fold change >|1.5|)

Additiional Data table S3 (separate file)

Table S3. Significantly differentially expressed genes in liver. Workbook tabs indicate conditions for lists of DEG from liver (FDR-corrected p-value < 0.05, fold change >|1.5|)